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(54) Title: TARGETED NITRIC OXIDE PATHWAY OR NITRIC OXIDE SYNTHASE MODULATION

(57) Abstract

The present invention is directed to targeted agents capable of modulating a nitric oxide pathway or nitric oxide synthase to achieve a therapeutic effect. Some preferred targeted agents include a targeting portion capable of delivering the agent to a target site and an effector portion capable of modulating a nitric oxide pathway or nitric oxide synthase at the target site. The present invention also provides methods of modulating a nitric oxide pathway or nitric oxide synthase to achieve a therapeutic effect in a target cell population.

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TARGETED NITRIC OXIDE PATHWAY OR NITRIC OXIDE SYNTHASE MODULATION

Technical Field of the Invention

The present invention is directed to targeted modulation of a nitric oxide pathway or nitric oxide synthase to achieve therapeutic goals. More specifically, the present invention is directed to targeted control of nitric oxide producing and utilizing cellular mechanisms that are mediated by nitric oxide synthase or other catalysts as well as other nitric oxide synthase-mediated reactions.

Background of the Invention

Many cell types synthesize and/or utilize nitric oxide. For example, nitric oxide has been identified as endothelium-derived relaxing factor (EDRF) which acts as a potent vasodilator by relaxing vascular smooth muscle through the activation of soluble guanylate cyclase and the cGMP cascade. Brain and liver cells also employ active nitric oxide pathways.

Generally, nitric oxide is synthesized from L-arginine in a reaction mediated by the enzyme nitric oxide synthase. Nitric oxide synthase mediates other cellular reactions such as the conversion of L-arginine to L-citrulline. A plurality of nitric oxide synthase isozymes have thus far been identified. Nitric oxide synthase may be induced by cytokines, such as tumor necrosis factor and interleukin-1, in vascular smooth muscle cells, for example. Extended duration nitric oxide production has been achieved in cells by degradation of S-nitroso-nucleophiles over time.

Stenosis following vascular reconstruction (restenosis) has been shown to be largely the result of proliferation of arterial smooth muscle cells in the intima in response to endothelial denudation. Many possible contributors to the proliferative effect have been investigated, such as platelet aggregation, platelet-derived

growth factor (PDGF) and the lack of EDRF. Loss of vascular response is also observed in septic shock and during tumor therapy using cytokines.

It would therefore be desirable to tap the therapeutic potential of nitric oxide and nitric oxide synthase through the controlled manipulation of the metabolic pathways in which those moieties play a role.

Summary of the Invention

The present invention is directed to agents capable of modulating a nitric oxide pathway of a target cell population to achieve a therapeutic effect. Such agents include a targeting portion capable of delivering the agent to a target site and an effector portion capable of modulating a nitric oxide pathway at the target site. Another aspect of the present invention includes agents capable of modulating nitric oxide synthase, thereby providing modulation of receptor-mediated reactions in which the enzyme plays a role. Optionally, additional agents useful in the practice of the present invention include free (i.e., untargeted) molecules which act in a systemic manner. The agents of the present invention may be formed as conjugates or fusion proteins. Agents of the present invention may also be composed of two or more separately administrable moieties. In one embodiment, the first exhibits a targeting portion-ligand composition and the second has an anti-ligand-effector moiety structure. In another embodiment, nitric oxide synthase-targeting portion is administered, followed by effector moiety administration.

Preferred targeting portions of the present invention include monoclonal antibodies or other targeting proteins directed to cells with constitutive or inducible nitric oxide pathways. Exemplary of such preferred targeting portions include monoclonal antibodies or other targeting

proteins directed to vascular smooth muscle cells, corpora cavernosa smooth muscle cells, endothelial cells, brain cells, and liver cells as well as monoclonal antibodies or other targeting proteins directed to tumor cells.

Preferred effector portions of agents of the present invention include moieties capable of modulating a nitric oxide pathway. Preferred effector portions include nitric oxide synthase or moieties capable of mediating the action of nitric oxide synthase enzyme(s) (e.g., L-arginine modified with imidazole, acetylene or allyl functional groups). Exemplary preferred effector portions of agents of the present invention include nitrate compounds, L-arginine, poly-L-arginine, N^G-hydroxy-L-arginine or polymers thereof, N^G-hydroxy-N^G-methyl-L-arginine, other arginine analogs, glutamic acid, poly-glutamic acid, N-methyl-D-aspartate, poly-N-methyl-D-aspartate, glucocorticosteroids, heme, non-heme iron or compounds which facilitate site-specific delivery of molecular oxygen or superoxide anion.

Other preferred agents of the present invention include antibodies or other targeting proteins that have undergone sulfhydryl modification to form S-nitroso moieties or, for example, L-arginine nitrogen modification to form nonoate equivalents. Such modified proteins, e.g., derivitized L-arginine, lysine, serine, tyrosine (with the latter two examples having undergone hydroxy modification) or the like, provide targeted nitric oxide species for an extended period of time, thereby achieving longer duration target site-specific agonist effects upon the nitric oxide pathway. The present invention also provides methods of modulating a nitric oxide pathway to achieve a therapeutic effect in a target cell population. Such methods include administration of a therapeutically effective amount of an agent including a targeting portion capable of delivering the agent to a target site and an effector portion capable of

modulating the nitric oxide pathway at the target site. Another embodiment of the present invention involves methods of modulating nitric oxide synthase. Methods of the present invention additionally or alternatively include administration of a free molecule to achieve systemic nitric oxide pathway or nitric oxide synthase modulation. In addition, the present invention contemplates a methodology involving administration of a targeting portion-ligand (e.g., avidin or streptavidin) molecule followed by administration of a anti-ligand (e.g., biotin)-effector portion (e.g., nitric oxide donor moiety). In this manner, the target cell population is "pre-targeted" by the targeting portion-ligand moiety to achieve enhanced localization of the anti-ligand-effector portion to those sites. Other methods of the present invention involve administration of nitric oxide synthase directly or indirectly bound to a targeting portion and subsequent administration of a nitric oxide synthase modulator.

Therapeutic effects which can be achieved-by methods of the present invention are those that involve a constitutive or an inducible target cell nitric oxide pathway. Exemplary therapeutic effects include treatment of restenosis, treatment of homeostasis of endothelial cells, cancer treatment and treatment of septic shock.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the nitric oxide pathway exhibited by vascular smooth muscle cells.

Fig. 2 is a schematic representation of a two-step therapeutic method embodiment of the present invention for restenosis treatment.

Fig. 3A graphically depicts experimental data regarding protein synthesis inhibition capability of Calphostin C with respect to vascular smooth muscle cells.

Fig. 3B graphically depicts experimental data regarding protein synthesis inhibition capability of methyl arginine with respect to vascular smooth muscle cells.

Fig. 3C graphically depicts experimental data regarding protein synthesis inhibition capability of nitro arginine with respect to vascular smooth muscle cells.

Fig. 3D graphically depicts experimental data regarding protein synthesis inhibition capability of nitroglycerin with respect to vascular smooth muscle cells.

Fig. 4A graphically depicts experimental data regarding DNA synthesis inhibition capability of sodium nitroprusside with respect to vascular smooth muscle cells.

Fig. 4B graphically depicts experimental data regarding DNA synthesis inhibition capability of nitroglycerin with respect to vascular smooth muscle cells.

Detailed Description of Preferred Embodiments

Prior to describing preferred embodiments of the present invention, definitions of terms thought to be useful in the understanding the invention are set forth.

Targeting Portion: A molecule that binds to a defined population of cells. The targeting portion may bind a receptor, an enzymatic substrate, an antigenic determinant or other binding site present on the target cell population. Proteins, such as antibodies and other polypeptides, are used throughout the specification as prototypical examples of targeting portions of the present invention.

Effector Portion: A molecule or combination of molecules capable of modulating a constitutive or an inducible nitric oxide pathway.

Analog: A molecule that is structurally similar to and exhibits the opposite modulating activity from the molecule of which it is an "analog."

Ligand/Anti-Ligand Pair: A member of a complementary/anti-complementary set of molecules that demonstrate specific binding, generally of relatively high affinity. Exemplary ligand/anti-ligand pairs include hapten/antibody, ligand/receptor and biotin/avidin or streptavidin. Biotin/avidin is used throughout the specification as a prototypical example of a ligand/anti-ligand pair.

Ligand: A relatively small, soluble molecule that exhibits rapid serum, blood and/or whole body clearance when administered intravenously in an animal or human.

Anti-Ligand: A moiety that demonstrates high affinity, and, preferably, multivalent binding of the complementary ligand. More preferably, the anti-ligand is large enough to avoid rapid renal clearance, and contains sufficient multivalency to accomplish crosslinking and aggregation of targeting portion-ligand conjugates.

Avidin: A compound capable of high affinity, multivalent or univalent binding of biotin, including avidin, streptavidin and derivatives and analogs thereof.

The present invention is directed to targeted agents capable of modulating a nitric oxide pathway to achieve a therapeutic effect. An illustrative nitric oxide pathway is shown in Fig. 1, with the reaction being susceptible to cytokine induction and L-arginine analog inhibition. Nitric oxide synthesis from L-arginine is modulated by nitric oxide synthase, an enzyme for which a plurality of isozymes have been identified. The presence of cytokines at nitric oxide synthesis sites (intercellular or intracellular sites where the reactants and the catalyst are found) therefore results in nitric oxide formation, while the presence of N-alkyl (as opposed to N-hydroxy) L-arginine analogs prevents such formation. Consequently, reactions for which nitric oxide is required (e.g., soluble guanylate

cyclase activation) do not occur or occur at a reduced rate (i.e., are down modulated) when L-arginine analogs are present. Such reactions occur or occur at a faster rate (i.e., are up regulated) when cytokines are present.

A preferred embodiment of a targeted agent of the present invention includes a targeting portion bound to an effector portion by covalent or non-covalent binding, with covalent binding preferred. Equivalent targeted agents of this embodiment of the present invention are fusion proteins including targeting and effector moieties. The targeting portion delivers the targeted agent to target cell sites, while the effector portion modulates a nitric oxide pathway at such sites. The targeting portion and the effector portion may be formed as separate molecules or be combined in a single molecule.

Targeting portions useful in the present invention include molecules capable of binding a defined population of cells, which cells exhibit a constitutive or an inducible nitric oxide pathway. Targeting portions which facilitate agent internalization into the target cells are preferred for effector portions requiring metabolic processing to impact the level of nitric oxide in the cell. For effector portions that spontaneously decompose to nitric oxide (i.e., do not require metabolic processing to achieve an increased nitric oxide level), internalization capability is of less significance, because the highly lipophilic and diffusible nitric oxide molecule can reach intracellular effector sites. Preferred targeting portions for use in the practice of the present invention include moieties capable of localizing to smooth muscle cells such as vascular smooth muscle cells, corpus cavernosum smooth muscle cells or the like, brain cells such as cerebellar cells, certain neurons or the like, liver cells such as hepatocytes and Kupffer cells or the like, cancer cells including melanoma, lymphoma,

adenocarcinoma, hepatoma or the like, endothelial cells, macrophages, platelets, fibroblasts, mesangial cells, neutrophils, renal collecting duct cells, intestinal cells such as intestinal muscle cells, intestinal neural cells or the like, chondrocytes or the like, for example. Exemplary of such targeting portions are antibodies or fragments thereof that localize to such target cells, avidin or streptavidin conjugates of such antibodies or fragments, receptor specific proteins or peptides, wherein such targeting portions can optionally be used in liposome, microparticle, nanoparticle or other appropriate dosage forms.

Effector portions of the present invention include molecules or combinations of molecules that are capable of modulating a nitric oxide pathway of cells to which the targeting portion of the targeted agent localizes. The term "modulation" encompasses both enhancement and inhibition of nitric oxide production or of reactions modulated by nitric oxide. Preferred effector moieties useful in the practice of the present invention include nitrate compounds, L-arginine, poly-L-arginine, arginine analogs (e.g., N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester, N^G-hydroxy-L-arginine, N^G-hydroxy-N^G-methyl-L-arginine or the like), excitatory amino acids and polymers thereof (e.g., glutamic acid, poly-glutamic acid, N-methyl-D-aspartate, poly-N-methyl-D-aspartate), glucocorticosteroids, corticosteroids, transforming growth factors, macrophage deactivating factor, cytokines such as interferon, tumor necrosis factor, interleukin-1, interleukin-2 and lipopolysaccharide, aminoguanidine, heme, non-heme iron or compounds which facilitate site-specific delivery of molecular oxygen or superoxide anion, superoxide ion scavengers, GMP

phosphodiesterase inhibitors, histamine, vasopressin, epinephrine, serotonin, acetylcholine (ACh), ADP, alpha-adrenergic agonists, thrombin and bradykinin, for example. Of these exemplary effector molecules L-arginine, N^G-hydroxy-L-arginine, glutamic acid, N-methyl-D-aspartate and polymers thereof, heme, non-heme iron, cytokines and GMP phosphodiesterase inhibitors, for example, exhibit potentiating effects, while glucocorticosteroids, other arginine analogs (including N^G-hydroxy-N^G-methyl-L-arginine, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester or the like), corticosteroids, transforming growth factors, macrophage deactivating factor and aminoguanidine, for example, exhibit inhibitory effects.

The present invention is also directed to targeted agents capable of modulating nitric oxide synthase to achieve a therapeutic effect. Fig. 1 is illustrative of a typical nitric oxide synthase-mediated reaction. An inducer/potentiator of nitric oxide synthase production or activity causes an increase in production of nitric oxide (Fig. 1) or products of other reactions mediated by nitric oxide synthase. In contrast, an inhibitor of nitric oxide synthase production or activity causes a decrease in the production of nitric oxide (Fig. 1) or other nitric oxide synthase-mediated reaction product.

Embodiments of this aspect of the present invention involve a targeting portion bound to an effector portion by covalent or non-covalent binding, with covalent binding preferred. Equivalent targeted agents of this embodiment of the present invention are fusion proteins including targeting portions and effector portions. The targeting portion of such agents of the present invention delivers the agent to

target cell sites, while the effector portion modulates a nitric oxide synthase catalyzed reaction at such sites.

Targeting portions useful in this embodiment of the present invention include molecules capable of binding a defined population of cells, which cells exhibit receptor-mediated reactions in which nitric oxide synthase plays a role. Targeting portions which facilitate agent internalization into the target cells are amenable to the practice of the present invention. Also, targeting portions specific for extracellular epitopes are useful in the practice of the present invention, as a result of the highly diffusible nature of nitric oxide. Preferred targeting portions for use in the practice of this embodiment of the present invention include moieties capable of localizing to smooth muscle cells such as vascular smooth muscle cells, corpus cavernosum smooth muscle cells or the like, brain cells such as cerebellar cells, certain neurons or the like, liver cells such as hepatocytes, Kupffer cells or the like, certain cancer cells including melanoma, lymphoma, adenocarcinoma, hepatoma or the like, endothelial cells, macrophages, platelets, fibroblasts, mesangial cells neutrophils, polymorphonuclear granulocytes, renal collecting duct cells, intestinal cells such as intestinal muscle cells and intestinal neural cells, chondrocytes, or the like. Exemplary of such targeting moieties are antibodies or fragments thereof that localize to such target cells, avidin or streptavidin conjugates of such antibodies or fragments, receptor specific proteins or peptides, optionally delivered in liposome, microparticle nanoparticle or other appropriate dosage forms.

Effector portions of this embodiment of the present invention include molecules or combinations of molecules that are capable of modulating nitric oxide synthase in cells to which the targeting portion of the targeted agent localizes.

The term "modulation" encompasses both enhancement and inhibition of nitric oxide synthase production or activity. Preferred effector moieties useful in the practice of this embodiment of the present invention include glucocorticosteroids, L-arginine modified with imidazole, acetylenic or allylic functionalities, L-arginine, poly-L-arginine, L-arginine analogs (e.g., N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester or the like), excitatory amino acids and polymers thereof (e.g., glutamic acid, poly-glutamic acid, N-methyl-D-aspartate, poly-N-methyl-D-aspartate) and aminoguanidine for example. Of these exemplary effector molecules L-arginine and poly-L-arginine, for example, exhibit potentiating effects, while modified L-arginine, L-arginine analogs, aminoguanidine and glucocorticosteroids are exemplary of moieties that exhibit inhibitory effects.

Some effector molecules useful in targeted agents of the present invention exhibit reversible effector properties, while others exhibit irreversible effector properties. More specifically, "reversible" and "irreversible" effector moieties may exhibit either a potentiating or an inhibitory effect on the target cell. Administration of an oppositely acting effector molecule results in reversal of the effect exerted by a reversible effector. In contrast, the metabolic modulation of an irreversible effector is not reversed by subsequent administration of an oppositely acting effector moiety. Exemplary reversible effector molecules are modified arginine, such as arginine substituted with functionalities including imidazole, acetylene, cyclopropyl as well as nitro. Exemplary irreversible effector molecules are N-methyl arginine, N-allyl arginine and the like.

For example, arginine modified with an imidazole functionality may be targeted by an appropriate antibody and internalized within a target cell. The modified arginine exhibits an inhibitory effect on nitric oxide synthase. Upon administration of L-arginine, for example, which has a potentiating effect on nitric oxide synthase, the inhibitory effect of the imidazole-modified arginine is reversed. If the first administered targeted agent inhibitory effector moiety is allyl arginine or methyl arginine subsequent administration of L-arginine will not appreciably reverse the inhibitory effect, however.

Another effector portion useful in targeted agents of the present invention includes a molecule which reacts with or otherwise deactivates nitric oxide. Such effector portions have an antagonistic effect on nitric oxide pathways. Exemplary effector portions of this embodiment of the present invention are heme, non-heme iron, compounds that facilitate the site-specific delivery of molecular oxygen, superoxide ion, or reducing equivalents such as thiols, ascorbate and the like.

Targeting portions and effector portions of targeted agents of the present invention may be bound by any convenient mechanism therefor. Preferably, the link between the targeting portion and the effector portion is cleavable through the action of intracellular processes or in response to the intracellular environment for embodiments of the present invention involving effector portions that require metabolic processing to impact the target cellular nitric oxide level. For embodiments including effector portions that spontaneously decompose to nitric oxide (i.e., do not require metabolic processing to achieve an increased nitric oxide level), internalization capability is of less significance, because the highly lipophilic and diffusible nitric oxide molecule can reach intracellular effector sites.

For example, proteinaceous targeting portions may be directly linked to amino acid-based effector portions through a peptide bond by procedures that are known in the art. Peptide bonds are cleavable by endogenous peptidases of target cells, thereby releasing the effector molecule from the agent in those cells.

Alternatively, such moieties may be directly linked by other covalent or non-covalent bonds or indirectly linked through cross-linkers such as heterobifunctional cross-linking compounds, ligand-anti-ligand pairs or the like. Both the indirect and direct coupling methods employ reactive groups, such as hydroxyl, amino, amido, or sulfhydryl groups, in an effector molecule and in a corresponding targeting portion as well as in the optional cross-linking agent. Bonds, such as a peptide bond, disulfide bond, thioester bond, amide bond, thioether bond, or the like, can be employed for this purpose.

Appropriate linking techniques are known in the art. The linker methodology is influenced by the choice of targeting portion and effector portion, by physical properties of the targeted agent (e.g., shelf life stability), biological properties of the targeted agent (e.g., half-life in cells and blood and intracellular compartmentalization route) and the like.

Targeting portions and effector portions of targeted agents of the present invention may be formed using fusion protein genetic engineering technology. Fusion proteins are hybrid proteins generated using recombinant DNA technology. A fusion protein is translated from messenger RNA as one continuous polypeptide chain, with the protein or peptide components joined together by peptide bonds. A recombinant DNA fusion sequence corresponding to a conjugate of the claimed invention may be cloned and expressed according to standard procedures. Briefly, the recombinant

DNA fusion sequence is inserted in vitro into an expression vector capable of replication in a particular host microorganism. Typically, the expression vector is derived from a plasmid or virus. See Old and Primrose, Principals of Gene Manipulation, 2d ed., University of California Press, pp. 104-17, 1981; PCT Patent Application Publication No. WO 86/00528; United States Patent Nos. 4,599,311 and 4,704,362; and British Patent No. GB 2,119,804.

Another aspect of the present invention involves administration of a free (i.e., untargeted or unbound to a targeting portion) effector molecule to achieve systemic modulation of a nitric oxide pathway or nitric oxide synthase. Exemplary effector molecules useful in the practice of this aspect of the present invention include arginine, arginine analogs such as N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester or the like, nitrate compounds such as sodium nitroprusside, nitrosothiols, nonoates and nitroglycerin and amyl nitrate, isosorbide diuate, hydralazine and pentaerythritol tetranitrate, for example.

Such free effector molecule administration may be conducted alone or, as preferred, in combination with a targeting portion-effector portion targeted agent of the present invention. Free effector/targeted agent administration can be serial or simultaneous. In this manner, both targeted and systemic modulation is achieved.

An additional embodiment of the present invention involves nitric oxide donor moieties. Preferred nitric oxide donor moieties spontaneously degrade under physiological conditions to form nitric oxide. Such moieties include molecules that have undergone sulfhydryl modification to form S-nitroso substituted molecules, L-arginine modification to form nonoate equivalents or the like. Sulfhydryl

modification may be accomplished, for example, by disulfide bond reduction to form thiol functionalities or by utilization of endogenous thiol groups on the agent. For example, one or more targeting portion thiol groups are derivitized with nitric oxide gas or $NaNO_2$ to yield a moiety of the following structure: protein-S-NO. For example, derivitization of cysteine forms such a moiety. Alternatively protein-O-nitroso (e.g., tyrosine or serine derivitization) or protein-N-nitroso (e.g., lysine derivitization) can be formed.

Such modified moieties may act as both targeting portions and effector portions and, therefore, as targeted agents in accordance with the present invention. For S-nitroso substituted antibodies, for example, such modification may be conducted upon endogenous sulfhydryls or sulfhydryls introduced into the antibody structure by the exposure of the antibody to a reducing agent. Provided the modification does not interfere with the target cell binding, S-nitroso substituted antibodies constitute targeted agents of the present invention.

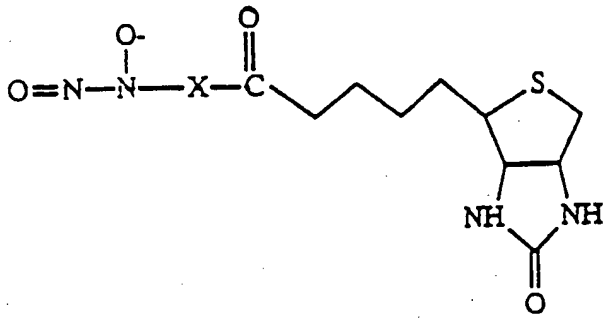
Alternatively, S-nitroso moieties may be introduced into a physiologically acceptable sulfhydryl containing molecule to generate an effector portion capable of delivery by a targeting portion. Nonoate groups can be introduced into L-arginine, L-lysine, serine, tyrosine or like molecules to generate an effector portion capable of delivery by a targeting portion. The modified molecules or nitric oxide donor containing effector portion targeted agents deliver a nitric oxide producing species (through degradation of the donor moiety) at the target site for an extended time period (e.g., from about 1 hour to about 24 hours). In this manner, sustained agonist effects upon the target nitric oxide pathway are achieved.

An example of this inventive concept involves the targeting of endothelial cells which exhibit a constitutive nitric oxide pathway. Elevated levels of nitric oxide cause vasodilation. At lower levels, nitric oxide serves to inhibit platelet activation and neutrophil adherence and to combat the cytotoxic effects of superoxide radicals. Ischemia and reperfusion interfere with proper endothelial cell function with respect to nitric oxide production. Addition of S-nitroso compounds or targeted S-nitroso effector molecules, for example, provides a source of nitric oxide to the target cell, thereby protecting ischemic-reperfused endothelial tissues from the action of superoxide radicals. Exemplary targeting portions useful in the practice of this embodiment of the present invention are S-nitroso or nonoate modified endothelial cell targeting antibodies or proteins or endothelial targeting antibodies or proteins bound to S-nitroso or nonoate containing effector molecules.

Additional embodiments of the present invention provide targeting portion-ligand and effector portion-anti-ligand conjugates or fusion proteins for serial administration to a mammalian recipient. Targeting portion-ligand is administered first to permit localization to the target site or removal from the recipient by the relevant metabolic pathway therefor. Effector portion-anti-ligand is subsequently administered (with a time lag between administrations ranging from about 4 hours to about 72 hours). Preferably, the effector portion-anti-ligand is either bound at the target site by ligand-anti-ligand interaction with the targeting portion-ligand or is rapidly cleared from the recipient (with a clearance time ranging from about 5 minutes to about 2 hours). Preferred targeting moiety structures include antibodies, proteins or peptides, or dosage forms such as liposomes or solid supports (e.g.,

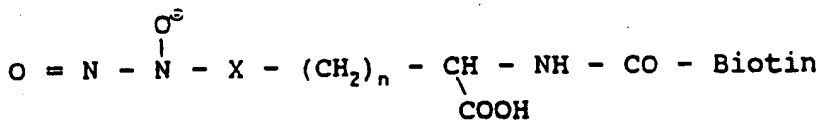
polymers) capable of specifically localizing to a target cell population. Preferred effector portions useful in these embodiments of the present invention are nitric oxide donor molecules or an isozyme of the nitric oxide synthase enzyme.

For example, an avidinylated monoclonal antibody (i.e., ligand-targeting portion) is administered to a recipient and localizes to a target cell population. The avidinylated targeting portion thus provides a receptor for a biotinylated nitric oxide donor molecule (i.e., anti-ligand-effector portion) to achieve sustained release of nitric oxide at the target site. At about 4 to about 72 hours post-avidinylated monoclonal antibody administration, biotinylated nitric oxide donor molecule is administered, with an exemplary molecule being of the following structure:



wherein X is a spacer group selected to impart certain desired physical or biological properties to the biotinylated effector molecule. Exemplary X groups are $-HN-(CH_2)_n-NH-$, $-RN-(CH_2)_n-NH-$ (where R is an alkyl group), spermine, multicarboxylate compounds (e.g., diethylene triamine pentaacetic acid, often abbreviated as DTPA), dextran or the like, which impart sustained release of nitric oxide when targeted or rapid clearance properties when not localized to target cells. Preferably, n ranges from 1 to about 5.

Another exemplary compound of the present invention is structured as follows:



where X is NH, O or S. These molecules impart sustained release of NO when targeted or rapid clearance when not localized to target cells.

Another example of this inventive concept involves avidin-targeting portion administration followed by administration of biotinylated nitric oxide synthase. This embodiment of the present invention is also practicable by a nitric oxide synthase effector portion/targeting portion conjugate, fusion protein or other dosage form. These targeted agents of the present invention localize nitric oxide synthase enzymatic activity at a target site following administration, for example, by the preferred injection or implantation routes. Targeted release of nitric oxide is then achieved by administration of the starting material for nitric oxide synthase-mediated nitric oxide production, L-arginine, preferably by an oral or an injection route. Nitric oxide release by this procedure is not characterized by the adverse systemic consequences observed for moieties such as drugs or toxins, because of the lipophilicity and reactivity of nitric oxide. More specifically, nitric oxide is a highly lipophilic molecule that easily diffuses into cells located adjacent to or in the vicinity of the targeting moiety. In addition, nitric oxide is highly reactive and, therefore, does not appreciably diffuse into the systemic circulation prior to being inactivated.

As indicated above, a preferred ligand-anti-ligand pair useful in the practice of the present invention is avidin-biotin. Either avidin or biotin can constitute the

ligand or anti-ligand of the agents of the present invention, with biotin being preferred as the anti-ligand. Other ligand-anti-ligand pairs, including lectin/carbohydrate, DNA/zinc fingers and the like, are also employable in accordance with the present invention.

Ligand-targeting protein conjugates, fusion proteins and other dosage forms may be prepared in accordance with known coupling, genetic engineering and related techniques. Anti-ligand-effector portion conjugates, fusion proteins and other dosage forms may also be prepared using known techniques.

Cell types exhibiting a constitutive or an inducible nitric oxide pathway are susceptible to the practice of the present invention. Exemplary target cell types are smooth muscle cells such as vascular smooth muscle cells, corpus cavernosum smooth muscle cells or the like, brain cells such as cerebellar cells, certain neurons or the like, liver cells such as hepatocytes, Kupffer cells or the like, certain cancer cells including melanoma, lymphoma, adenocarcinoma, hepatoma or the like, endothelial cells, macrophages, platelets, fibroblasts, mesangial cells neutrophils, polymorphonuclear granulocytes, renal collecting duct cells, intestinal cells such as intestinal muscle cells and intestinal neural cells, chondrocytes or the like.

Also, cell types exhibiting receptor-mediated reactions in which nitric oxide synthase plays a role are susceptible to the practice of the present invention. Exemplary cell types are vascular smooth muscle cells, brain cells, liver cells, certain cancer cells, endothelial cells and the like. Cells exhibiting nitric oxide synthase controlled, NMDA receptor mediated events, for example, are amenable to the practice of the present invention.

For example, vascular smooth muscle cells employ a metabolic pathway to produce nitric oxide, a molecule which

is capable of activating soluble guanylate cyclase and the cGMP cascade. This nitric oxide pathway is schematically shown in Fig. 1. Other cell types are impacted by cGMP modulation through manipulation of a nitric oxide pathway. For example, platelet aggregation is inhibited by elevated cGMP levels (corresponding to elevated nitric oxide levels). Likewise, Na^+ absorption of renal duct collecting cells is inhibited by elevated cGMP (nitric oxide) levels.

In the Fig. 1 vascular smooth muscle embodiment, nitric oxide synthesis is induced by cytokines, such as tumor necrosis factor and interleukin-1. Consequently, such cytokines are effector portions useful in the practice of the present invention to modulate the nitric oxide pathway of vascular smooth muscle cells to produce nitric oxide. The lack of nitric oxide has been implicated as a contributor to the proliferation of arterial smooth muscle cells that is characteristic of restenosis. Stimulation of nitric oxide synthesis diminishes or obviates the problem of nitric oxide deficiency, thereby promoting homeostasis of smooth muscle cells.

Also, nitric oxide synthase is competitively inhibited by L-arginine analogs, such as N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester or the like. Consequently, cytokine-induced effects can be inhibited by L-arginine analogs as well. This fact forms one of the bases for the two-step vascular smooth muscle treatment protocol shown in Fig. 2. In the first step, activation and/or action of nitric oxide synthase is inhibited by an administered antibody-nitric oxide synthase inhibitor conjugate (e.g., an L-arginine analog-antibody conjugate). As a result, production of nitric oxide is decreased, thereby inhibiting nitric oxide-

potentiated events such as cGMP cascade activation, platelet disaggregation, smooth muscle relaxation, and cytotoxicity.

In step 2, a monoclonal antibody-nitric oxide synthase inducer conjugate (e.g., poly-L-lysine or glutamic acid or N-methyl-D-aspartate to antibody conjugate) or free nitric oxide donor (e.g., a nitrate analog, such as sodium nitroprusside, nitrosothiols, nonoates and nitroglycerin and amyl nitrate, isosorbide diuate, hydralazine and penta-erythritol tetranitrate) is administered, respectively generating targeted or systemic nitric oxide synthase induction. In this manner, nitric oxide (EDRF) is produced and supplied to vascular smooth muscle cells, thereby reducing the proliferation thereof and other effects including those shown in Fig. 2. Preferably, step 2 is conducted with a time lag ranging from about 5 minutes to about 72 hours following step 1.

Monoclonal antibody targeting portions useful in the practice of this embodiment of the present invention localize to chondroitin sulfate proteoglycans (CSPGs) synthesized by vascular smooth muscle cells and pericytes. A discrete portion of the 400kD proteoglycan complex CSPG molecule, an N-linked glycoprotein having an apparent molecular weight of about 250kD, is especially preferred as a target for the targeting portion of this aspect of the present invention. An exemplary targeting portion useful in this aspect of the present invention involves a NR-AN-01 monoclonal antibody (a subculture of NR-ML-05).

The monoclonal antibody designated NR-ML-05 binds a 250kD CSPG synthesized by melanoma cells (Morgan et al., U.S. Patent No. 4,897,255). Smooth muscle cells and pericytes also synthesize a 250kD CSPG as well as other CSPGs. NR-ML-05 binding to smooth muscle cells has been disclosed (Fritzberg et al., U.S. Patent No. 4,879,225). Monoclonal antibody NR-ML-05 and subculture NR-ML-05 No. 85-

41-4I-A2, freeze # A2106, have both been deposited with the American Type Culture Collection, Rockville, MD and granted Accession Nos. HB-5350 and HB-9350, respectively. NR-ML-05 is the parent of, and structurally and functionally equivalent to, subclone NR-AN-01, disclosed herein.

Other targeting portions associating with the 400kD CSPG target and other epitopes in that target are also useful in the practice of this embodiment of the present invention. The amino acid residues involved in the multi-point kinetic association of the NR-AN-01 monoclonal antibody with a CSPG marker antigenic epitope, (i.e., the amino acids constituting the complementarity determining regions), are determinable by computer-assisted molecular modeling and by the use of mutants having altered antibody binding affinity. The binding-site amino acids and three dimensional model of the NR-AN-01 antigen binding site serve as a molecular model for constructing functional equivalents, e.g., short polypeptides ("minimal polypeptides") that have binding affinity for a CSPG epitope synthesized by vascular smooth muscle cells and pericytes. As used herein "minimal polypeptide" refers to a sequence of at least six amino acids in length.

Three-dimensional modeling is also useful to construct other functional equivalents that mimic the binding of NR-AN-01 to its antigenic epitope, e.g., "mimetic" chemical compounds that mimic the three-dimensional aspects of NR-AN-01 binding to its epitope in a CSPG target antigen. As used herein, the term "mimetic" refers to an organic chemical polymer constructed to achieve the proper spacing for binding to the amino acids of an NR-AN-01 CSPG target synthesized by vascular smooth muscle cells or pericytes.

Human monoclonal antibodies or "humanized" murine antibody are also useful as targeting portions in accordance with the present invention. For example, murine monoclonal antibody may be "humanized" by genetically recombining

nucleotide sequence encoding the murine Fv region (i.e., containing the antigen binding sites) or the complementarity determining regions thereof with the nucleotide sequence encoding a human constant domain region and an Fc region, e.g., in a manner similar to that disclosed in European Patent Application No. 0,411,893 A2. Humanized targeting portions are recognized to decrease the immunoreactivity of the antibody or polypeptide in the host recipient, permitting an increase in the half-life and a reduction in the possibility of adverse immune reactions.

Targeted agents of the present invention may be administered in any convenient manner therefor. For example, an infusion catheter may be employed to deliver agents to certain target sites, such as vascular smooth muscle cells. Other routes of administration also find utility in the practice of the present invention. Exemplary administration routes are injection by the intravenous, intralymphatic, intrathecal, or other intracavity routes, oral administration, implantation and the like, depending primarily upon the target site and the structure of the targeted agent.

Targeted agents of the present invention are administered in such amounts as to deliver a therapeutically effective amount of effector molecule to target sites. Appropriate administered doses depend on a variety of factors that are largely patient specific. The components of the targeted agent used also impact dose amounts in ways that are known to or routinely ascertainable by practitioners in the art. In general, targeted agent is administered to mammals at a dose ranging between 1.0 mg and 1.0 g, depending upon the physiological characteristics of the patient and the ailment involved. A practitioner in the art is capable of identifying an appropriate dose and administration route for a given recipient with a given ailment.

Certain cancer cell types are characterized by or cause an accumulation of effector cells, such as macrophages, in the vicinity thereof. Such effector cells employ a nitric oxide pathway. Nitric oxide has been observed to have tumor suppressive properties. Consequently, effector cell nitric oxide production is manipulable by administration of targeted agents of the present invention directed to macrophage target cells. Increased target cell nitric oxide production caused by the targeted delivery of a nitric oxide synthesis potentiating effector portion results in a tumor cell suppression therapeutic benefit.

Alternatively and preferably, nitric oxide donor containing targeted agents or modified molecules provide a sustained release source of nitric oxide or a nitric oxide synthesis potentiator (e.g., a cytokine) to tumor cell sites. Exemplary targeting portions useful in this aspect of the present invention include antibodies, peptides, proteins and the like directed to tumor cell antigens. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to cancer cells are also useful in accordance with the present invention. Such moieties may be identified and constructed or isolated in accordance with known techniques. An exemplary targeting portion useful in the practice of the present invention is NR-LU-10, a monoclonal antibody reactive with a pancarcinoma glycoprotein of approximately 40,000 dalton molecular weight.

Septic shock, which results from exposure to bacterial endotoxin and manifests in cardiovascular collapse, may be addressed with targeted agents of the present invention. Nitric oxide is present in elevated amounts in septic patients exhibiting hemodynamic compromise. Production of nitric oxide results in reduced vascular contractility. Inhibition of nitric oxide production

reverses the hypotension. As a result, useful effector portions for this embodiment of the present invention modulate the nitric oxide pathway in a manner opposite to that indicated above for cancer treatment. More specifically, antagonist rather than agonist effector molecule function is utilized. Consequently, L-arginine analogs are exemplary effector molecules useful in the practice of this aspect of the present invention. Exemplary targeting portions useful in this embodiment of the present invention include NR-AN-01 or an endothelial cell targeting portion. Targeting portions useful in this aspect of the present invention include antibodies, peptides, proteins and the like. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to endothelial cells are also useful in accordance with the present invention.

Nitric oxide is a mediator of inflammation. Chronic, non-infectious inflammation results in the stimulation of nitric oxide production. Consequently, modulation of nitric oxide at sites of inflammation impacts the operation of the inflammation cascade. Effector moieties capable of either stimulation or inhibition of nitric oxide production find utility in the practice of the present invention to, respectively, augment or diminish the activity of the inflammation cascade. Exemplary effector moieties are L-arginine nitrates, nonoates, nitrosothiols, hydroxy-arginine and like molecules as discussed herein (potentiating nitric oxide production) as well as L-arginine analogs, alkyl arginine, polymers thereof and like molecules as discussed herein (inhibiting nitric oxide production). Targeting portions useful in the practice of this embodiment of the present invention include those directed to antigens that are up regulated during the inflammation process such as adhesion antigens. Also, hepatic cells can be protected

from inflammation related injury by enhanced nitric oxide synthesis, thereby rendering targeting portions that localize to such cells useful in the practice of the present invention. Exemplary targeting portions are galactosyl proteins (which target hepatocytes) and mannosyl proteins, for example. Targeting portions useful in this aspect of the present invention include antibodies, peptides, proteins and the like. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell populations are also useful in accordance with the present invention.

As indicated above, nitric oxide exerts protective effects on organs such as the liver. Some of these effects may be the result of nitric oxide interaction with superoxide ion or prevention of microvascular perfusion deficits. The liver exhibits an inducible nitric oxide pathway where maximum induction of nitric oxide synthesis is accomplished by multiple inducing moieties (e.g., interferon, interleukin-1, tumor necrosis factor and lipopolysaccharide). Such cytokines or other effector moieties capable of inducing nitric oxide production in hepatic cells are preferred for use in liver therapy embodiments of the present invention. Targeting portions of agents used in this aspect of the present invention localize to hepatocytes. Exemplary of such targeting portions are galactosyl proteins, for example. Targeting portions useful in this aspect of the present invention include antibodies, peptides, proteins and the like. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell population are also useful in accordance with the present invention.

Like hepatocytes, macrophages exhibit a cytokine-inducible nitric oxide synthase. Macrophages process the antigens of intercellular pathogens and present such antigens

to T-cells for recognition and cell-mediated destruction. Nitric oxide produced by macrophages modulates proliferative and cytolytic responses of T-cells. The presence of nitric oxide suppresses T-cell proliferation and cytolytic processes mediated by T-cells. Consequently, L-arginine analogs such as N^G-monoomethyl-L-arginine can be targeted to macrophages to down modulate nitric oxide synthesis if T-cell proliferative or cytolytic activity is desired. Exemplary T-cell targeting agents useful in the practice of the present invention are monoclonal antibodies, fragments thereof and other specific receptor binding moieties. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell population are also useful in accordance with the present invention.

Glutamate has been implicated in neurodegeneration disorders such as ischemic hypoxic insults, Alzheimer's disease and Huntington's disease. Glutamate neurotoxicity has been found to be mediated by nitric oxide. Inhibition of nitric oxide production by, for example, L-arginine analogs such as N^G-nitro-L-arginine and N^G-monomethyl-L-arginine results in a decrease in glutamate receptor mediated cell death. A major source of the nitric oxide that mediates neurotoxicity is nitric oxide synthase containing neurons. Such neurons are therefore appropriate targets for localization by the targeting portions of this aspect of the present invention. Exemplary targeting portions are monoclonal antibodies, fragments thereof and other specific receptor binding moieties. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target-cell population are also useful in accordance with the present invention.

It has been demonstrated in endothelial cells that nitric oxide production is inhibited by agents that generate

the superoxide ion. Superoxide ion scavengers, such as superoxide dismutase, ascorbate, glutathione or the like, may be employed as effector molecules in targeted agents of the present invention designed to prevent or diminish superoxide inhibition of the nitric oxide pathway. Targeting portions useful in the practice of this embodiment of the present invention localize to cells which exhibit a constitutive or inducible nitric oxide pathway and are exposed to agents generating oxide radicals such as the superoxide radical. Endothelial cells, smooth muscle cells, macrophages, brain cells and the like are exemplary sets of target cells. Exemplary targeting agents therefore include monoclonal antibodies, fragments thereof and other specific receptor binding moieties. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell population are also useful in accordance with the present invention.

Hyperaemia, thought to limit acid injury to the mucosa caused by acid back diffusion through a disrupted gastric mucosal barrier, is an increase in gastric mucosal blood flow. Nitric oxide mediates gastric mucosal vasodilation. An increase in nitric oxide production results in an increase in mucosal blood flow. This phenomena is believed to be the result of the operation of the nitric oxide pathway of neurotransmitters responsible for submucosal and mucosal vasodilation. Consequently, L-arginine effector molecules or other nitric oxide synthesis potentiators may be targeted to such neurotransmitters by an appropriate targeting portion. Exemplary targeting portions are monoclonal antibodies, fragments thereof and other specific receptor binding moieties. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell population are also useful in accordance with the present invention.

Nitric oxide is a mediator of relaxation of corpus cavernosum resulting from nonadrenergic noncholinergic neurotransmission. The presence of nitric oxide therefore promotes penile erection. Nitric oxide synthesis potentiators, such as L-arginine, can be employed as effector portions of agents of the present invention designed to achieve penile erection. Corpus cavernosum smooth muscle cells are the appropriate target for the targeting portion used in this embodiment of the present invention. Exemplary targeting portions are monoclonal antibodies, fragments thereof and other specific receptor binding moieties. Minimal peptides, mimetic organic chemical compounds, human or humanized monoclonal antibodies and the like that localize to relevant target cell population are also useful in accordance with the present invention.

EXAMPLE 1

The ability of several therapeutic agents to inhibit DNA synthesis and protein synthesis in vascular smooth muscle cells was tested. ^{3}H-leucine and ^{3}H-thymidine uptake and cytotoxicity assays were conducted in accordance with the following protocols.

^{3}H-leucine uptake: Vascular smooth muscle cells (B054 baboon smooth muscle cells) at 40,000 cells/ml were seeded in sterile 24 well plates at 1 ml/well. The plates were incubated overnight at 37°C, CO_2, 95% air in a humidified atmosphere (saturation). Log dilutions of the therapeutic agent being tested were incubated with the vascular smooth muscle cells for 5 minutes or 24 hours. Samples of the therapeutic agent were diluted in DMEM: F-12 media (Whittaker Bioproducts, Walkersville, Maryland) with 5% fetal bovine serum (FBS, Gibco) and 5% Serum Plush® (JRH Biological). Following therapeutic agent incubation,

the solution was aspirated, and 1 ml/well of 0.5 microcurie/ml ^{3}H-leucine in leucine-free DMEM (Dulbecco's Modified Eagle's Medium) with 5% Serum Plush® was added. The plates were re-incubated overnight at 37°C, 5% CO_2 in a humidified atmosphere. The cells were visually graded using an inverted microscope using a scoring scale to determine viability and cell number. The 1 to 3 grade is based upon percent of cell viability and number compared to control wells, with 3=100%, 2=70%-100% and 1=0%-70%. A record of this scoring assisted in determining the immediate cytotoxic effect of the therapeutic agent. The media was then aspirated, and the cells were washed twice with cold 5% TCA. 400 microliters of 0.2M NaOH was added per well, and the plates were incubated for two hours at room temperature on a rotating platform. 200 microliters per well of the cell solution was transferred into plastic scintillation vials (Bio-Rad Laboratories), and 4 milliliters of Bio-Safe® II liquid scintillation fluid was added prior to vortexing. Vials were counted on a Beckman LS2800 liquid scintillation counter interfaced with Beckman "Data Capture" software for conversion to a Lotus 1-2-3® file and analysis using Lotus 1-2-3®.

^{3}H-thymidine uptake: Vascular smooth muscle cells were incubated in complete media with 5% FBS (Gibco) overnight at 37°C in a humidified, 5% CO_2 environment in sterile 24 well plates. The media was aspirated from the wells and serum free media supplemented with growth factors (DMEM: F-12 basal media supplemented with growth factor cocktail, catalog number I1884, which contains insulin (5 micrograms/ml), transferrin (5 micrograms/ml) and sodium selenite (5 nanograms/ml), available from Sigma Chemical, St. Louis, Missouri) was added. Cells were incubated in this media for 24 hours. For a 5 minute therapeutic agent exposure, log dilutions of the therapeutic agent were

incubated with the cells in complete media. After 5 minutes and media aspiration, 1 ml/well of 1.0 microcurie/ml ^{3}H-thymidine dispersed in complete media was added. The 24 hour exposure involved incubation of the cells with 1 ml/well of 1.0 microcurie/ml of ^{3}H-thymidine dispersed in complete media and log dilutions of therapeutic agent being tested. In both exposure trials, the cells were then incubated overnight at 37°C in a humidified, 5% CO_2 environment. The cells were visually scored for viability and cell number. Cells were washed and prepared for transfer into plastic scintillation vials as described for the ^{3}H-leucine protocol. Vials were counted on a Beckman LS2800 liquid scintillation counter interfaced with Beckman "Data Capture" software for conversion to a Lotus 1-2-3® file and analysis using Lotus 1-2-3®.

These protocols are amenable to use with other therapeutic agents as well as with other target cell populations, especially adherent monolayer cell types.

Morphological Cytotoxicity Evaluation-Pulsed Exposure: Vascular smooth muscle cells were seeded at 4.0 x 10^4 cells/ml media/well on a commercially prepared four well slide (Nunc, Inc., Naperville, Illinois). Enough slides were seeded to accommodate two pulsed exposure lengths (5 minutes and 24 hours) and prescribed increment evaluation points (24 hours to 128 hours). All slides were run in duplicate to reveal any assay anomalies. Nitroglycerin was diluted in the same media used in the ^{3}H-leucine and ^{3}H-thymidine assays. Each four well slide was concentration bracketed to one log greater concentration (well "B"), one log lower concentration (well "D") of the minimal effective concentration (well "C"), as determined by the ^{3}H-leucine and ^{3}H-thymidine assays. As a control for normal morphology, one well (well "A") was left untreated (media only). Incubation took place in a 37°C, 5% CO_2 humidified incubator. After

each of the two (5 minutes and 24 hours) exposure points, nitroglycerin media was aspirated from each well, including the untreated-well. One milliliter of fresh media was then added to replace the aspirated media. Re-incubation followed until each of the incremented evaluation points were achieved. At those points, the media was aspirated and subsequently replaced with 1 ml of 10% neutral buffered formal in for one hour to allow for proper fixation. These fixed slides were stained by hematoxylin (nuclear) and eosin (cytoplasmic) for morphologic evaluation and grading.

Results: Figs. 3A-3D and 4A-4B show exemplary results of ^{3}H-leucine and ^{3}H-thymidine studies for vascular smooth muscle target cells (B054 cells) exposed to various effector moieties. Fig. 3A indicates that Calphostin C inhibits protein synthesis of the target cell population, while Figs. 3B-3D indicate that methyl arginine, nitro arginine and nitroglycerin do not inhibit protein synthesis. Consequently, administration of Calphostin C will kill the target cells, while administration of the other effector portions will not. Figs. 4A and 4B indicate that sodium nitroprusside and nitroglycerin inhibit DNA synthesis. Consequently, administration of such moieties will result in target cell metabolic modulation (diminished proliferation). A comparison of Figs. 3D and 4B indicates that nitroglycerin administration results in metabolic modulation of the target cells without killing the target cells.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic

principles of the invention.

WHAT IS CLAIMED IS:

1. A targeted agent capable of modulating a nitric oxide synthase isozyme or a nitric oxide pathway to achieve a therapeutic objective with respect to a target cell population, the targeted agent comprising:
a targeting portion capable of delivering the targeted agent to the target cell population; and
an effector portion integral with or capable of coupling to the targeting portion and capable of modulating a nitric oxide synthase isozyme or a nitric oxide pathway associated with the target cell population.

2. A targeted agent according to claim 1 wherein the targeting portion and effector portion are in conjugate form and are directly coupled or coupled through a heterobifunctional linker or a ligand-anti-ligand pair.

3. A targeted agent according to claim 2 wherein the targeting portion comprises an antibody, an antibody fragment, a protein complementary to a target cell receptor, another peptide capable of delivering the targeted agent to the target cell population or a functional equivalent thereof.

4. A targeted agent according to claim 2 wherein the targeting portion localizes to a target cell population that utilizes a constitutive or inducible nitric oxide pathway selected from the group comprising vascular smooth muscle cells, corpora cavernosa smooth muscle cells, endothelial cells, brain cells and liver cells.

5. A targeted agent according to claim 4 wherein the targeting portion comprises NR-AN-01 and the target cell population comprises vascular smooth muscle cells.

6. A targeted agent according to claim 2 wherein the targeting portion localizes to a cancerous target cell population selected from the group consisting of a

pancarcinoma antibody or fragment thereof that recognizes a membrane glycoprotein of approximately 40,000 daltons.

7. A targeted agent according to claim 2 wherein the effector portion potentiates nitric oxide synthesis.

8. A targeted agent according to claim 7 wherein the effector portion is selected from the group comprising L-arginine, polymers thereof glutamic acid, polymers thereof, N-methyl-D-aspartate, polymers thereof, heme, non-heme iron, cytokines, GMP phosphodiesterase inhibitors, superoxide ion scavengers and functional equivalents thereof.

9. A targeted agent according to claim 2 wherein the effector portion inhibits nitric oxide synthesis.

10. A targeted agent according to claim 9 wherein the effector portion is selected from the group comprising glucocorticosteroids, arginine analogs, corticosteroids, transforming growth factors, aminoguanidine, macrophage deactivating factor and functional equivalents thereof.

11. A targeted agent according to claim 10 wherein the effector portion is N^G-hydroxy-N^G-methyl-L-arginine, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, N^G-amino-L-arginine, N^G-cyclopropyl-L-arginine, N^G-amino-L-homoarginine, N^G-nitro-L-arginine methyl ester and functional equivalents thereof.

12. A targeted agent of claim 2 wherein the effector portion comprises a nitric oxide synthase isozyme or a functional equivalent thereof.

13. A targeted agent according to claim 1 wherein the targeting portion and effector portion are of integral structure and the integral structure comprises a nitric oxide donor moiety derivitized molecule that is capable of localizing to a target cell population.

14. A targeted agent according to claim 13 wherein the nitric oxide donor moiety comprises a S-nitroso group, a nonoate equivalent or a functional equivalent thereof.

15. A targeted agent according to claim 1, comprising two separately administrable components, wherein the targeting portion is bound to a ligand and the effector portion is bound to an anti-ligand that is complementary to the ligand.

16. A targeted agent of claim 15 wherein the ligand-anti-ligand pair comprises avidin-biotin, streptavidin-biotin or a functional equivalent thereof.

17. A method of achieving a therapeutic objective with respect to a target cell population, which method comprises administration to a recipient of a targeted agent comprising:
a targeting portion capable of delivering the targeted agent to the target cell population; and
an effector portion integral with or capable of coupling to the targeting portion and capable of modulating a nitric oxide synthase isozyme or a nitric oxide pathway associated with the target cell population.

18. A method according to claim 17 which further comprises administration of a free effector portion to achieve systemic and targeted modulation of a nitric oxide synthase isozyme or a nitric oxide pathway associated-with the target cell population.

19. A method according to claim 17 wherein the target cell population is selected from the group comprising vascular smooth muscle cells, corpora cavernosa smooth muscle cells, endothelial cells, brain cells and liver cells.

20. A method according to claim 17 wherein the therapeutic objective is treatment of restenosis, the target cell population is vascular smooth muscle cells, the targeting agent localizes to such vascular smooth muscle cells and the effector portion is a nitric oxide synthesis potentiator.

21. A method according to claim 17 wherein the therapeutic objective is treatment of septic shock, the

target cell population is vascular smooth muscle cells or endothelial cells, the targeting agent localizes to vascular smooth muscle cells or endothelial cells and the effector portion is a nitric oxide synthesis inhibitor.

22. A method according to claim 17 wherein the therapeutic objective is modulation of inflammation, the target cell population is inflammation-related antigens bearing cells, the targeting agent localizes to the target cell population and the effector portion is a nitric oxide synthesis inhibitor if a decrease in inflammation cascade function is desired or a nitric oxide synthesis potentiator if an increase in inflammation cascade function is desired.

23. A method according to claim 17 wherein the therapeutic objective is protection of the liver from oxidation damage, the target cell population is liver cells, the targeting agent comprises a galactosyl protein and the effector portion comprises a plurality of nitric oxide synthesis potentiators.

24. A method according to claim 17 wherein the therapeutic objective is proliferation or cytolytic activity of T cells, the target cell population is macrophages, the targeting agent localizes to macrophages and the effector portion comprises a nitric oxide synthesis inhibitor.

25. A method according to claim 17 wherein the therapeutic objective is treatment of neurodegeneration disorders mediated by glutamate, the target cell population is neurons, the targeting agent localizes to neurons and the effector portion comprises a nitric oxide synthesis inhibitor.

26. A method according to claim 17 wherein the therapeutic objective is to diminish inhibition of a nitric oxide pathway, the target cell population cells exposed to oxide radicals or agents generating oxide radicals, the

targeting agent localizes to the target cell population and the effector portion comprises a oxide radical scavenger.

27. A method according to claim 17 wherein the therapeutic objective is hyperaemia treatment, the target cell population is neurotransmitters responsible for submucosal and mucosal vasodilation, the targeting agent localizes to the neurotransmitters and the effector portion comprises a nitric oxide potentiator.

28. A method according to claim 17 wherein the therapeutic objective is penile erection promotion, the target cell population is corpora cavernosa smooth muscle cells, the targeting agent localizes to the target cell population and the effector portion comprises a nitric oxide potentiator.

29. A method of achieving a therapeutic objective with respect to a target cell population, which method comprises administration of a targeted agent comprising:

a targeting portion capable of delivering the targeted agent to the target cell population; and

an effector portion integral with the targeting portion capable of modulating a nitric oxide synthase isozyme or a nitric oxide pathway associated with the target cell population by donating nitric oxide in a sustained release manner through degradation of the integral targeted agent.

FIG. 1

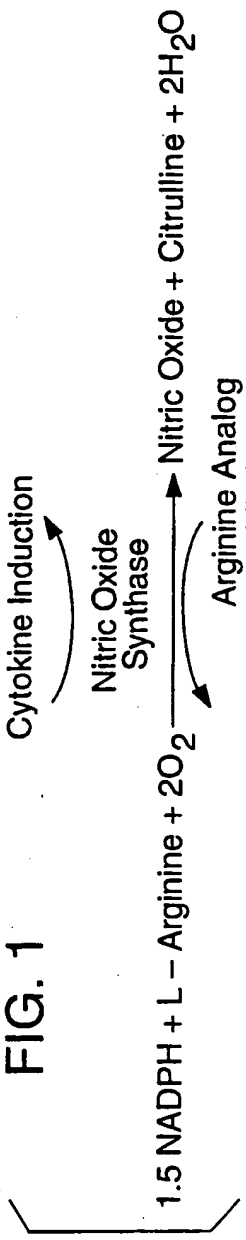

Cytokine Induction
Nitric Oxide Synthase
1.5 NADPH + L – Arginine + $2O_2$
Nitric Oxide + Citrulline + $2H_2O$
Arginine Analog Inhibition

FIG. 2

1 – Administer MAb – Nitric Oxide Synthase Inhibitor to Prevent
Nitric Oxide Activation
cGMP Cascade
and
2 – Administer MAb – Nitric Oxide Synthase Inducer or Free Nitric Oxide Synthase Inducer
Inducer To Achieve
Induction
Nitric Oxide Synthase
ADP Ribosylation
Inhibition of DNA Synthesis (Proliferation Reduction)
Nitric Oxide
Recombination with Activated Oxygen Species to form Cytotoxic Agents
Activation of Guanylate Cyclase

FIG. 3A

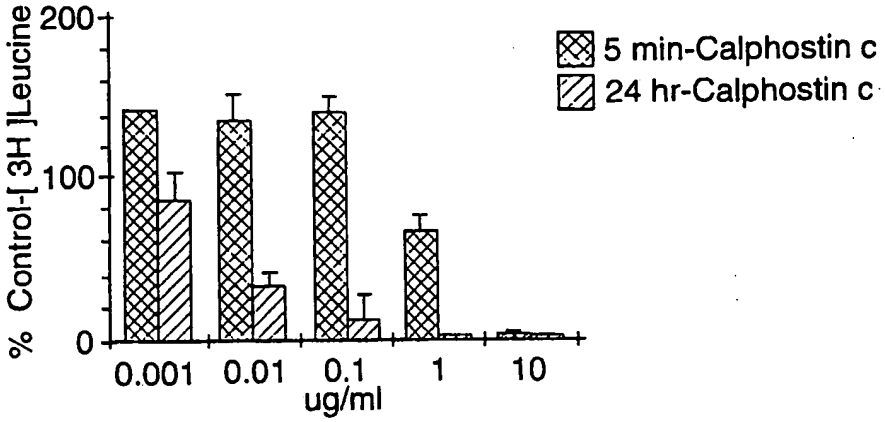


FIG. 3B

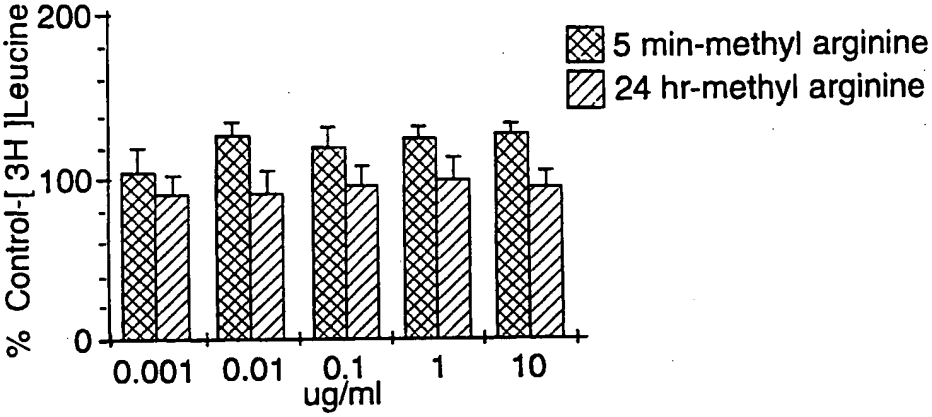


FIG. 3C

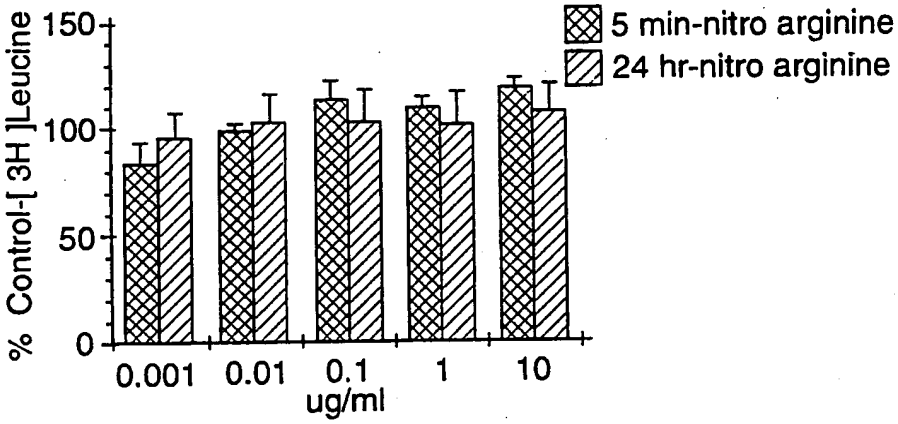


FIG. 3D

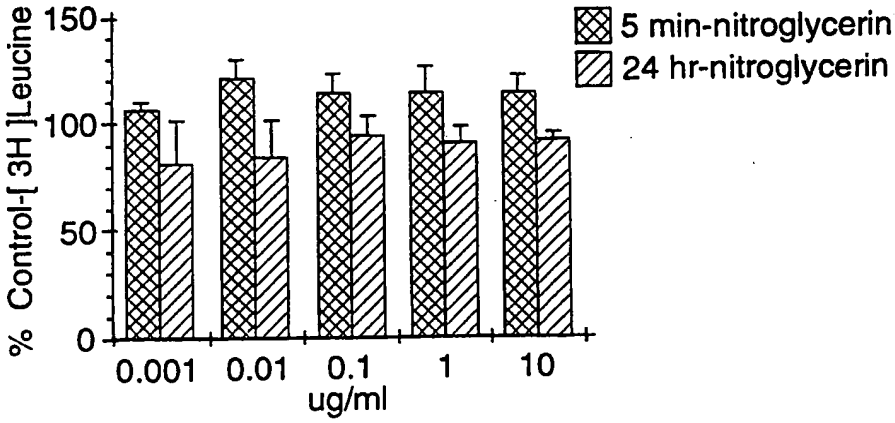


FIG. 4A

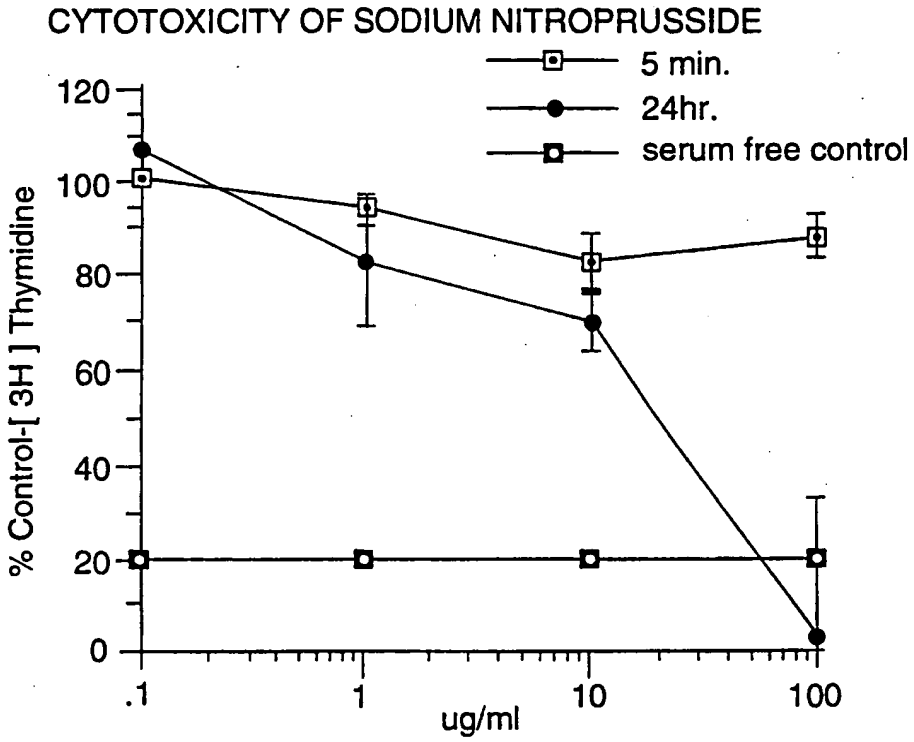
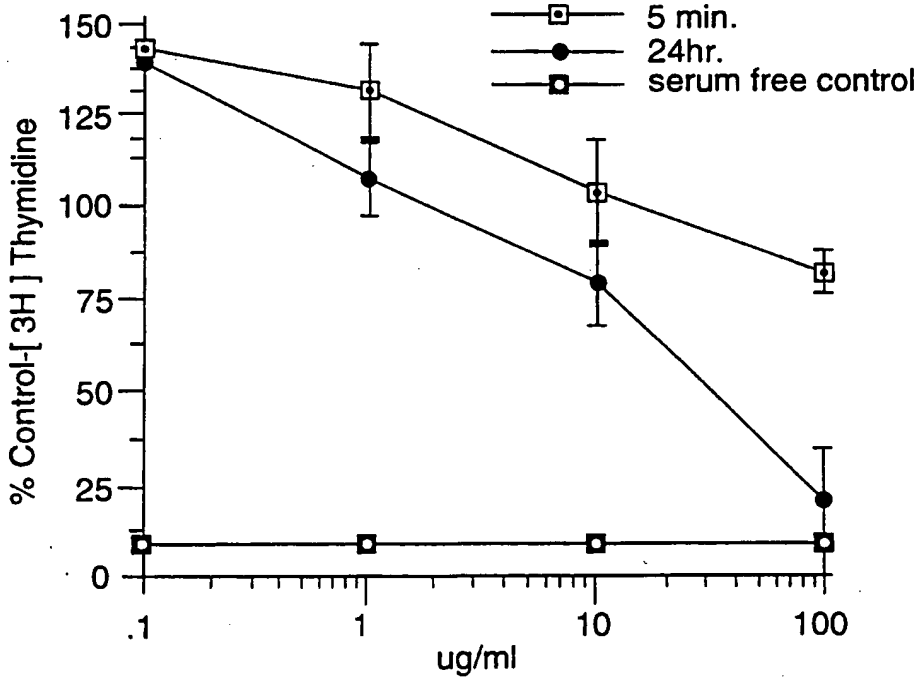

CYTOTOXICITY OF SODIUM NITROPRUSSIDE
5 min.
24hr.
serum free control
% Control-[3H] Thymidine
120
100
80
60
40
20
0
.1
1
10
100
ug/ml

FIG. 4B


CYTOTOXICITY OF NITROGLYCERIN (TRIDIL)
5 min.
24hr.
serum free control
% Control-[3H] Thymidine
150
125
100
75
50
25
0
.1
1
10
100
ug/ml

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00894

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 39/395, 31/00, 35/14; C07K 15/28; C12N 5/12
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.91; 514/1, 929; 530/388.8, 388.2, 391 1, 391.7, 391.9; 435/240.27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CHEM AB, EMBASE, DERWENT WPI, search terms: author names, nitric oxide, arginine, cells, vascular smooth cells, antibody

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FASEB Journal, Volume 3, issued January 1989, L.J. Ignarro, "Endothelium-derived nitric oxide: actions and properties", pages 31-36, see entire document.	1-8,15,16
Y	US, A, 4,867,973 (GOERS et al.) 19 September 1989, see entire document.	1-8,15,16
Y	US, A, 4,863,713 (GOODWIN et al.) 05 September 1989, see entire document.	15,16
Y	US, A, 4,981,979 (SIVAM) 01 January, 1991, see entire document.	6
Y	TIBS, Volume 14, issued December 1989, M.A. Marletta, "Nitric oxide: biosynthesis and biological significance", pages 488-492, see entire document.	6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MARCH 1994	Date of mailing of the international search report 11 APR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RON SCHWADRON Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00894

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.91; 514/1, 929; 530/388.8, 388.2, 391.1, 391.7, 391.9; 435/240.27